Compression of daily activity time in mice lacking functional Per or Cry genes

Daan, Serge; Comas, M.; Spoelstra, K.

Published in:
Chronobiology International

DOI:
10.3109/07420528.2014.885529

Publication date:
2014

Chronobiology International, 2014; 31(5): 645–654

ISSN: 0742-0528 print / 1525-6073 online
DOI: 10.3109/07420528.2014.885529

informa
healthcare

ORIGINAL ARTICLE

Compression of daily activity time in mice lacking functional *Per* or *Cry* genes

K. Spoelstra[1,2], M. Comas[1,3], and S. Daan[1]

[1]*Unit of Chronobiology, University of Groningen, Groningen, The Netherlands,* [2]*Department of Animal Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands, and* [3]*Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden*

The adjustment of daily activity time (α) to the varying night length in nocturnal creatures was one of the functions originally attributed to a putative dual oscillator structure of circadian pacemakers in mammals. In two experimental approaches, we tested whether this ability is compromised in mice with functional deletions of one of the four circadian clock genes. First, we tested the capability of α compression by long days in *mPer1*^{Brdm1} and *mPer2*^{Brdm1} mutant mice. When exposed to a full L:D 18:6 photoperiod, wild-type and *mPer1*^{Brdm1} mutant mice show compression followed by decompression of α in DD. *mPer2*^{Brdm1} mutant mice did not compress their activity time. The interpretation of these data is, however, complicated by masking due to light. We, therefore, embarked on a second experiment, exploiting skeleton photoperiods. The skeleton photoperiod was changed stepwise from 0 to 24 h, and *mCry1* and *mCry2* knockout mice were now included in the design. We observed clear and systematic compression of α in wild-type and *mCry1* and *mCry2* knockout mice. *mPer1*^{Brdm1} and *mPer2*^{Brdm1} mice both poorly entrained to the skeleton photoperiod. The single *mPer2*^{Brdm1} mutant mouse that did entrain did not show α compression. The results show that neither *mCry1* nor *mCry2* deletions compromise adjustment to day length, consistent with our earlier conclusions on period lengthening in constant light (Spoelstra & Daan, 2008). The *mPer2*^{Brdm1} mutant behaves aberrantly and appears not to respond to the delaying action of light in the late subjective day.

Keywords: Alpha compression, circadian clock, Cry1, Cry2, Per1, Per2, *Mus musculus*, photoperiod

INTRODUCTION

The daily timing of animal behavior is largely based on endogenous circadian programs. These programs have evolved to match the frequency of the light–dark cycle generated by the earth's rotation. The duration of the light and dark phase in this cycle varies greatly with season in locations remote from the equator. The question whether the endogenous program adjusts to these seasonal variations in day length has intrigued many chronobiologists. Circadian pacemakers are capable of storing information about the preceding photoperiod, as originally implied by after-effects of photoperiod on their spontaneous frequency *in vivo* (Pittendrigh & Daan, 1976a). *In vitro* studies have confirmed that isolated pacemakers reflect prior photoperiod in rhythms of melatonin production (sparrow pineal; Brandstätter et al., 2000), in spontaneous cFOS protein expression (rat SCN; Sumová & Illnerová, 1998), and in electrophysiological activity (hamster SCN; Jagota et al., 2000). At the behavioral level, the adjustment is observed in rodents by the compression and decompression of the daily activity time α (Pittendrigh & Daan, 1976a). As the night shortens in summer, the endogenously generated α shortens in nocturnal animals.

How pacemakers generate these patterns tuned to the photoperiod is of primary interest from a functional point of view. A simple proposition has been the idea that the pacemaker contains two components (M = morning; E = evening) which tend to phase-lock to dawn (M) and to dusk (E) as day length changes (Pittendrigh & Daan, 1976c). The two components were hypothesized to control the beginning (E) and end (M) of the daily activity time (α), such that these vary in the course of the year in tune with sunrise and sunset. The model has been expanded to the control of melatonin profiles in mammals (Illnerová, 1986), including humans (Wehr et al., 2001). The model did not specify

Submitted November 15, 2013, Returned for revision January 14, 2014, Accepted January 16, 2014

Correspondence: K. Spoelstra, Department of Animal Ecology, Netherlands Institute of Ecology (NIOO-KNAW), P.O. Box 50, 6700 AB Wageningen, The Netherlands. E-mail: K.Spoelstra@nioo.knaw.nl

whether the two components are active within individual rhythmic circadian neurons (pacers) in the pacemaker or distributed over different cells. Spatially separate E and M neurons have been found in *Drosophila* (Grima et al., 2004; Picot et al., 2007; Rieger et al., 2006; Stoleru et al., 2004, 2005, 2007; Yoshii et al., 2004). In the hamster, E and M peaks in SCN electrical activity appear after exposure to long photoperiods (Jagota et al., 2000). In the mouse, SCN separate regions couple under changing photoperiods to the onset and end of daily activity (Hazlerigg et al., 2005; Inagaki et al., 2007; Naito et al., 2008).

The question that can be addressed is how the genetic machinery of the pacemaker contributes to this seasonal adjustment. In this paper, we report experiments exploiting functional deletions of four genes – *Per1, Per2, Cry1, Cry2* – involved in this mechanism. The experiments have a bearing on a specific hypothesis based on the E–M pacemaking model, which stated that *Per1* and *Cry1* are specifically involved in the M-oscillator, and *Per2* and *Cry2* in the E oscillator (Daan et al., 2001). This hypothesis predicts that in the absence of one of these essential components the endogenous circadian program should remain entrained, but no longer follow dawn and dusk as in intact animals. The hypothesis could be rejected specifically for the *Cry* genes, although its predictions for the *Per* genes were confirmed in, first, the response to the intensity of continuous illumination (Spoelstra & Daan, 2008; Steinlechner et al., 2002), and, second, the phase response to brief light pulses (Pendergast et al., 2010; Spoelstra et al., 2004). If *Per1* and *Per2* indeed have a specific role in determining the morning and evening components, i.e. the offset and onset of daily activity, we might expect that functional deletion of either *Per1* or *Per2* disables α compression. Deletions of either *Cry* gene are not expected to cause such interference.

We first carried out an experiment using three photoperiods (LD 6:18; LD 12:12; LD 18:6) and assessed the daily activity time of wild-type mice as well as $mPer1^{Brdm1}$ and $mPer2^{Brdm1}$ mutant mice. The assay of α compression under the influence of long days is complicated by masking influences of light. Light suppressing activity of nocturnal rodents in long days may cause α to appear shorter than under long nights, even if the endogenous activity program has remained unchanged. The assessment of α in this protocol, therefore, heavily relies on the first few days in DD following photoperiod exposure. With pronounced masking, the gradual increase of α in DD still serves as evidence for compression followed by decompression. The problem of masking can further be avoided by the use of ''skeleton photoperiods'', where only two brief light pulses representing dawn and dusk are presented. The rodents *Peromyscus leucopus* and *Mesocricetus auratus* under a gradually shortening skeleton night compress α (Pittendrigh & Daan, 1976b). This compression cannot be due to masking. It could in principle be explained on the basis of the phase response curve, as the first pulse may delay the onset of activity and the second pulse may advance its offset, such that the duration of activity in between is reduced. However, compression went much further than predicted from the PRCs before the activity would ''jump over'' to the longer dark interval. Thus, the data were compatible only with the internal program coding for the length of the night interval (Pittendrigh & Daan, 1976c). We, therefore, used the same experimental approach to compress α in mice with functional deletions of the genes *Cry1, Cry2, Per1*, and *Per2* in a second experiment.

METHODS

Animals and maintenance

Male mice (*Mus musculus*) with functional deletions of *Cry1*, *Cry2, Per1,* or *Per2* genes and wild type as controls were used. The generation of $mCry1^{-/-}$, $mCry2^{-/-}$ has been described previously by van der Horst et al. (1999) and generation of $mPer1^{Brdm1}$, $mPer2^{Brdm1}$ mutant strains by Zheng et al. (1999, 2001). All mice were housed individually in $25 \times 25 \times 40$ cm cages, with food (Hope Farms standard rodent pellets, Arie Block, Woerden, the Netherlands) and water *ad libitum*, in a sound attenuated climatized room, at a temperature of 23 ± 1°C. Spontaneous locomotor activity was recorded with a running wheel (Ø 14 cm) in each cage, connected to an event recording system storing the number of wheel revolutions in 2 min intervals. All cages were placed at equal distance to the light source (white fluorescent tube light 36W/85) and received 480 ± 140 Lux (700 ± 200 mW/m^2) at the cage floor level.

Experiment 1

Seven $mPer1^{Brdm1}$ mutant mice (aged 315 ± 4 d), 8 $mPer2^{Brdm1}$ mutant mice (aged 317 ± 4 d) and 12 wild-type mice (aged 259 ± 5 d) were entrained to either LD 6:18, LD 12:12, or LD 18:6 for 20 d each. After entrainment, mice were released into constant dark (DD) for 12 d.

Experiment 2

Ninety male mice (aged 212 ± 3.3 d) were used: 15 homozygous $mCry1^{-/-}$ mutant mice, 15 homozygous $mCry2^{-/-}$ mutant mice, 15 wild-type control mice; 15 homozygous $mPer1^{Brdm1}$ mutant mice, 15 homozygous $mPer2^{Brdm1}$ mutant mice, and 15 wild-type mice. First, all mice were kept under LD 8:16 for 14 d, followed by LD 4:20 for 14 d, and then LD 2:22 for 32 d in order to have all mice stably entrained to a long night and short day. The 2 h of light were then substituted by two separate 60 min light pulses (skeleton photoperiod PPs 2) on day 64, marking the beginning (A) and end (B) of the day. A and B then gradually were moved apart in steps of 2 h every 14 d, until they merged again on day 206 and formed an L:D 2:22 full photoperiod again. The duration of the night was always symmetrically adjusted

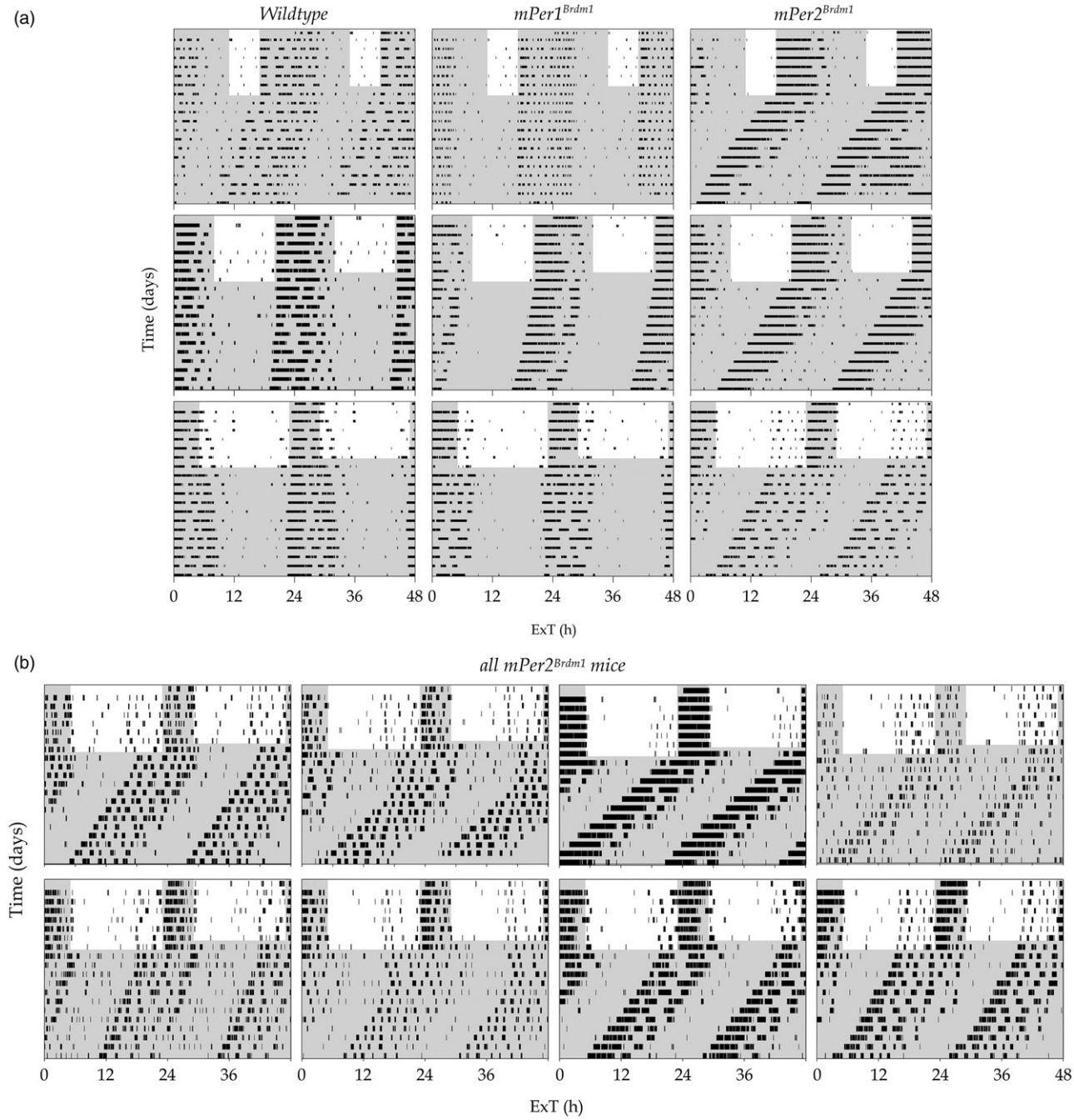

FIGURE 1. (A) Examples of double plotted actograms for wild type, *mPer1*^{Brdm1} and *mPer2*^{Brdm1} mice entrained under three different photoperiods (LD 6:18, LD 12:12 or LD 18:6) after which they were all released in DD. (B) Actograms obtained from all *mPer2*^{Brdm1} under LD 18:6. All mice failed to compress their activity to the dark phase; the onset of activity is halfway the light phase.

when skeleton photoperiod was changed. Finally, the mice were subjected to 12 d in DD. The whole light schedule is visualised in the example actograms in Figure 4.

Analysis

In both experiments, we defined the phase of the onset as the first 2 min interval where the activity count exceeded the average daily activity, starting from 12 h before the circular Center of Gravity (CoG; Kenagy, 1980). We defined the offset in a similar way by detecting the first interval exceeding the average daily activity count going backwards in time starting 12 h after the CoG. The duration of activity α is always defined as the time interval between the onset and the offset of activity.

In experiment 1, we calculated onset and offset for each individual separately per day. This was important in order to study the changes in α over consecutive days after the transition to DD.

In experiment 2, we first averaged the activity pattern for each individual over 10 d in each skeleton

photoperiod, skipping the first 2 d in order to avoid inclusion of transient daily patterns. Only animals that were clearly entrained were included. We then subjected the averaged pattern to the same analysis to find the average daily onset and end of activity for each mouse in each part of the protocol.

RESULTS

Experiment 1

Figure 1(A) shows examples of actograms of the wild-type, *mPer1*^{Brdm1} and *mPer2*^{Brdm1} mice in LD 6:18, LD 12:12, and LD 18:6, followed by release in DD. Patterns in LD 6:18 (upper row) are rather similar across genotypes, with activity concentrated in the first 10 h of darkness. In LD 12:12, again the phenotypes are similar with activity largely contained within the dark phase. After both short and equinoctial photoperiods, the activity time seems to be maintained in subsequent darkness (DD). In the long photoperiod (lower panels), the wild-type mouse has α compressed to within the short 6-h dark phase, and α expands gradually over several days in the subsequent DD. In contrast, the *mPer2*^{Brdm1} mouse maintains a long α in LD 18:6, such that activity onset phase leads lights-off by about 6 h. The activity during this long light episode is suppressed by masking, but the internal program is nonetheless clearly visible. This becomes even clearer when after release in DD the full ca 12 h α is immediately visible on the first day. The *mPer1*^{Brdm1} mouse has its α extending into the daytime in the morning after the short night and appears to resume its stable α from the first day in DD onwards. Figure 1(B) demonstrates that indeed none of the *mPer2*^{Brdm1} compressed activity in LD 18:6. Instead, all *mPer2*^{Brdm1} started their activity about 6 h before lights off, although with lower activity levels, in this long photoperiod. We quantified these patterns by computing for each animal and each day the onset and offset of activity, and then averaging these data per genotype for each day. The results are shown in Figure 2. For *mPer2*^{Brdm1} mice, the calculation does not correspond well with visual observations during the LD phase due to the suppression of activity levels by masking. This effect disappears, however, when mice are released in DD. Figure 2 shows that on the first day in DD the mean onset of activity starts from lights-off in wild type and *mPer1*^{Brdm1}, and from 6 h before lights-off in *mPer2*^{Brdm1}. In contrast, the mean offset of activity starts on the first day in DD from lights-off in wild type

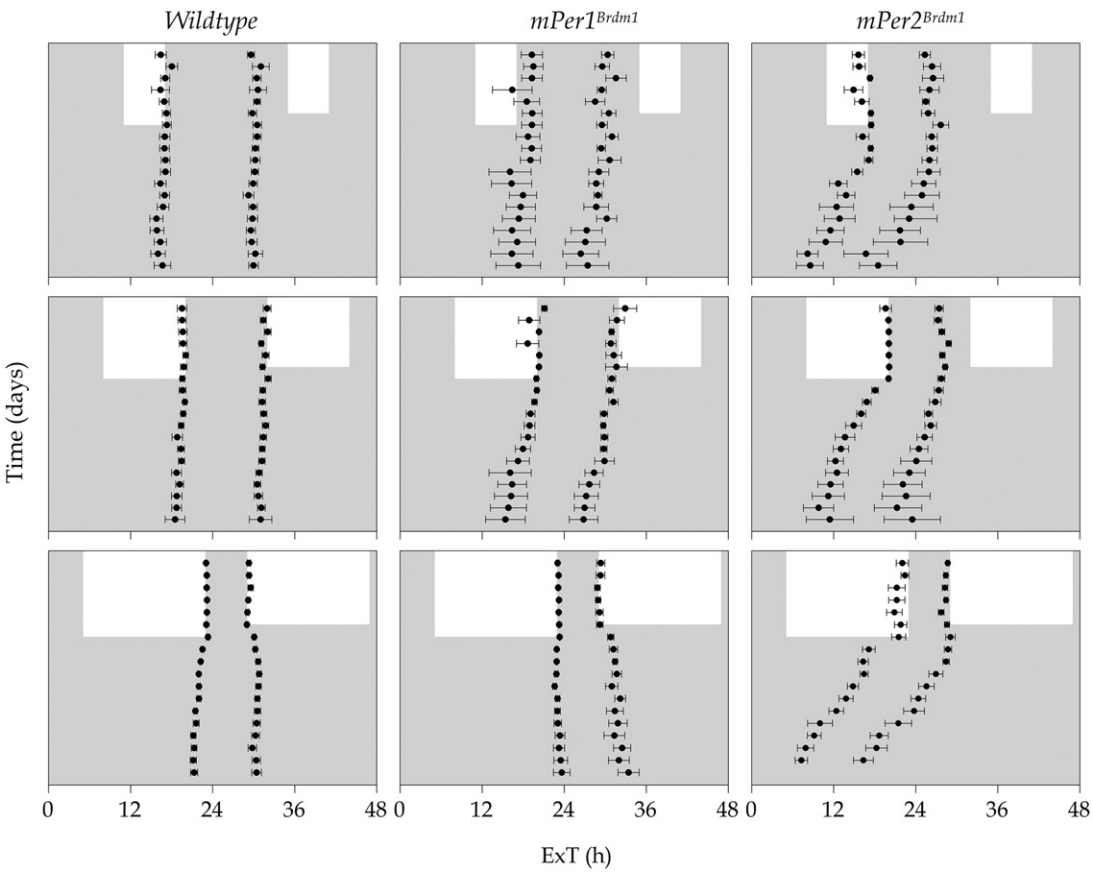

FIGURE 2. Average (±s.e.m.) position of onset and offset of activity of wild type, *mPer1*^{Brdm1} and *mPer2*^{Brdm1} during entrainment under LD 6:18 (upper row), LD 12:12 (middle row) or LD 18:6 (lower row) and subsequent release in DD.

and *mPer2*^{Brdm1}, and from ~2 h after lights-off in *mPer1*^{Brdm1}.

The temporal sequence of average α values per day following the transition from long photoperiod to DD is shown in Figure 3. This figure demonstrates the absence of decompression of α in DD in *mPer2*^{Brdm1} mice. Wild-type mice and *mPer1*^{Brdm1} have shorter α in DD. They seem to behave quite similarly. Wild-type mice significantly expanded α from 8 to 9 h in 10 d, demonstrating the after-effects of the short night. Decompression of α was less and non-significant in *mPer1*^{Brdm1} and absent in *mPer2*^{Brdm1} mice (Table 1).

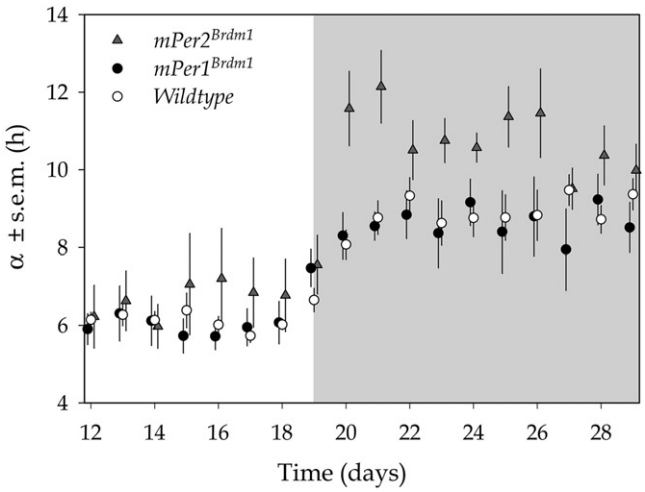

FIGURE 3. Average (± s.e.m.) of the daily α values of wild type, *mPer1*^{Brdm1} and *mPer2*^{Brdm1} during entrainment under LD 18:6 (white area) and subsequent release in DD (grey area).

Experiment 2

Figure 4 shows a complete actogram of one individual for each of the genotypes involved in the study, and, at the same time, illustrates the full protocol used. The endogenous free running circadian rhythm visible in the last part of the actogram is helpful in interpreting the pattern of entrainment in the circa 240 d before. We briefly discuss the patterns of entrainment in each of these examples, using the term BA for the dark interval between pulses B and A subsequently, and AB for the dark interval between pulses A and B (see also methods). The skeleton photoperiod is then AB + the duration of the two light pulses.

The wild-type mouse in Figure 4(A) has a normal τ of 23.9 h and its circadian system entrains by the delaying or decelerating effects of pulse B. It remains entrained with the activity in the interval BA, until this interval has been reduced from 22 to 8 h. Upon further reduction of BA to 6 h, it makes a backward delay jump to interval AB, probably induced by both pulse A and B now hitting the delay zone of the PRC. Compression of α is clearly visible from BA 12 to 8 h.

The *mCry1*$^{-/-}$ mouse in Figure 4(B) has a short circadian period (τ 22.6. h) and also entrains via delays. In this case, the single pulse B was not sufficient to generate the necessary delay alone, and entrainment results by the animal exploiting the delay effects of both A and B as long as these are sufficiently close together.

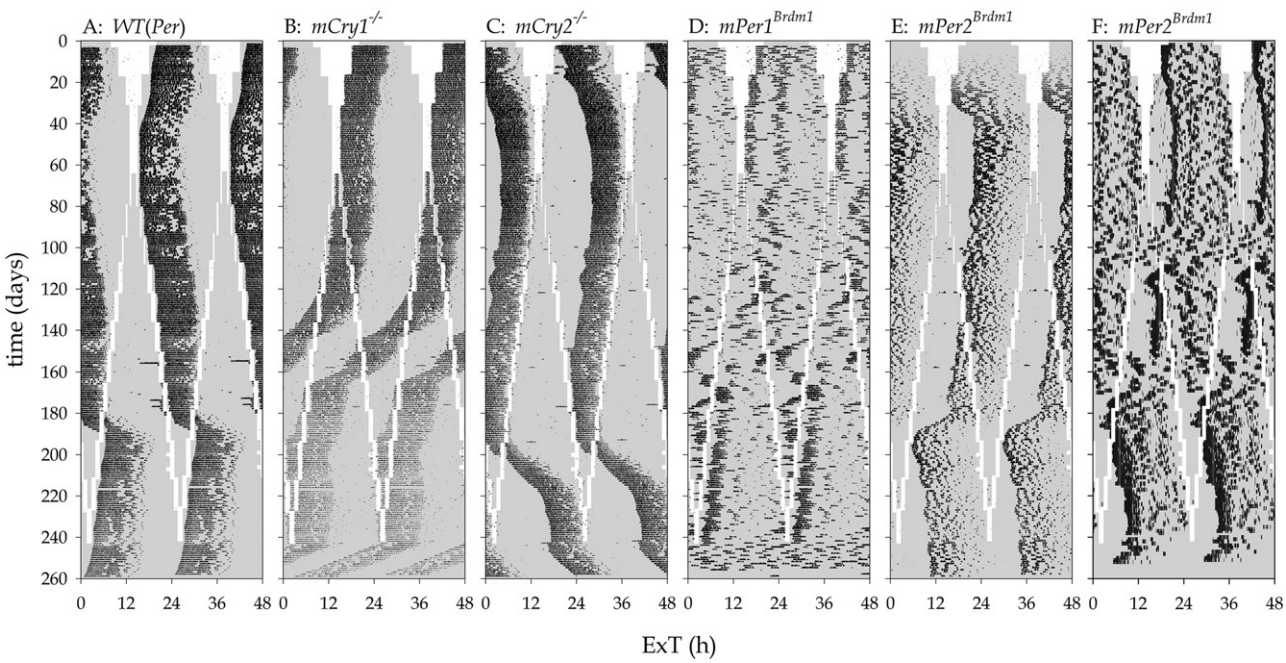

FIGURE 4. Examples of double plotted actograms for one animal from each of the genotypes studied under changing skeleton photoperiods. The light schedule is indicated by white vertical bars. (A) Wild type (29 out of 30 mice were rhythmic; 23 entrained by delays, 6 by advances); (B) *mCry1*$^{-/-}$ (11 out of 15 mice were rhythmic, all entrained by delays); (C) *mCry2*$^{-/-}$ (12 out of 15 mice were rhythmic, all entrained by advances); (D) *mPer1*^{Brdm1} (2 out of 15 mice were rhythmic, both entrained by delays); (E) the single rhythmic *mPer2*^{Brdm1} mouse (entrained by advances); (F) example of an arrhythmic *mPer2*^{Brdm1} mouse.

TABLE 1. Average of the activity duration (α) for wild-type, *mPer1*^{Brdm1} and *mPer2*^{Brdm1} mice in DD after having been entrained to LD 18:6. The first value shows the averaged α for the first day only, the second value shows the averaged α for the first 10 d in DD. A paired *t*-test shows that decompression of α was absent in *mPer2*^{Brdm1} mice and was reduced in comparison with decompression in wild type.

Genotype	Mean (SEM, *n*)	Paired *t*-test
Wildtype first day DD	8.07 (0.38; 12)	$T=-3.52$, df = 12
Wildtype average DD	8.94 (0.40; 12)	$p<0.01$
mPer1^{Brdm1} first day DD	8.30 (0.59; 7)	$T=-1.06$, df = 6,
mPer1^{Brdm1} average DD	8.64 (0.61; 7)	ns
mPer2^{Brdm1} first day DD	11.57 (0.95; 8)	$T=1.08$, df = 7,
mPer2^{Brdm1} average DD	10.74 (0.51; 8)	ns

By the time pulse B starts to hit the end of the activity time, i.e. the advance part of the PRC, entrainment can no longer be maintained and relative coordination ensues until the system could again lock on to the delaying action of the two pulses when they became sufficiently close in time again. The *mCry2*$^{-/-}$ mouse in Figure 4(C) has a long τ (24.5 h) and locks on with activity in the long dark interval, with pulse A generating sufficient advances for entrainment all the way from BA = 22 down to BA = 6 h. Compression of alpha is visible from BA = 10 down to BA = 6 h. At BA = 4 the jump to AB occurs via delaying transients, until again the system is entrained via advances. This actogram clearly suggests that *mCry2*$^{-/-}$ is capable of α compression.

The *mPer1*^{Brdm1} and *mPer2*^{Brdm1} mice used in this experiment were characterized by poor expression of rhythmicity. The *mPer1*^{Brdm1} mutant in Figure 4(D) shows evidence of circadian rhythmicity in some parts of the record, but not in all, and there is certainly no α visible or measurable on which to base any conclusion. Only one individual out of 15 *mPer2*^{Brdm1} did entrain to the skeleton photoperiod, and its activity pattern is shown in Figure 4(E). The animal was continuously entrained by the advancing effect of pulse A. It clearly maintained α throughout, without any compression, until activity started well before the BA interval of 8 h, as though there was no delaying effect of pulse B whatsoever. When B moved yet closer to A (BA = 6 h), the pulses apparently had a strong advancing effect together and activity jumped over by advancing into interval AB.

In Figure 5, the onsets and offsets of activity are averaged for wild type, *mCry1*$^{-/-}$ and *mCry2*$^{-/-}$ mice relative to the skeleton photoperiods, and separately for wild-type mice entraining by delays and advance. Records during which mice were not stably entrained, including those showing relative coordination, were excluded. Wild-type mice showed a dichotomy in behavior related to whether animals had an endogenous period τ shorter than 24 h in DD, or a τ longer than 24 h, and were separated accordingly. Wild-type mice entraining by delays (Figure 5A) all remained entrained with their activity onset locked on to the dusk pulse (i.e., the light pulse originally occurring at the initial time of lights off), as predicted for animals with short τ (Pittendrigh & Daan, 1976c). Entrainment broke down with a ψ jump to the other interval when PPs exceeded 16 h (Figure 5A). The *mCry1*$^{-/-}$ mice all entrained by delays, but here the activity onset remained exclusively locked on to the prior dawn pulse (Figure 5C). This is related to the fact that *mCry*$^{-/-}$ mice have a much shorter τ in DD than wild-type mice, and tend to have an earlier phase in entrainment. No phase jump occurred in this group because they had activity in the lengthening interval from scratch. At intermediate PPs from PPs 8 till 18, the Zeitgeber became too weak for stable entrainment for most animals.

Figure 5(B) combines the data for all six wild-type mice entraining via advances. The activity end locked on to the original dawn pulse. At PPs 14, the onset of activity collided with the dusk pulse, resulting in temporal entrainment by delays by the dusk pulse. The ψ jump occurred when PPs exceeded 16 h. All *mCry2*$^{-/-}$ mice had essentially the same pattern (Figure 5D). Here the ψ jump occurred yet later, around PPs 20. In Figure 5(G and H), we show the predicted onset phase (ψ) on the basis of τ and the PRC measured by Spoelstra et al. (2004).

On the basis of the onsets and ends of activity under all conditions as seen in Figure 5, we calculated the duration of activity time α (corresponding to the length of the horizontal bars in Figure 5), and plotted these against skeleton photoperiod in Figure 6. These plots show that the initial average group α, unrestricted by skeleton photoperiod varied from circa 10 (*mCry2*$^{-/-}$) till circa 13 h (*mCry1*$^{-/-}$), with the wild-type mice in between. Compression of α took place in the range of PPs between 10 and 16, just prior to the ψ jump. This appeared to occur also in the *mCry2*$^{-/-}$ knockouts. The *mCry1*$^{-/-}$ mice did not show α compression.

DISCUSSION

The prominent result from the full photoperiod experiment is that activity time α is compressed into the short night of summer photoperiods in wild-type mice and possibly in *mPer1*^{Brdm1} mouse mutants, but not in *mPer2*^{Brdm1} mice. This reflects the after effects of photoperiod on activity time. Compression may result from a change in the pacemaker output but it may also emerge from strong suppression of activity due to the masking effects of light. These two causes can be distinguished from the gradual decompression in DD following entrainment. This decompression was clear and significant in wild-type mice and not in the two mutants (Figure 3). While this might suggest that the *mPer1* mutant also has lost the facility of adjustment to photoperiod, we cannot be certain, since wild-type and *mPer1*^{Brdm1} mutants did not significantly differ in α values on the first day in DD.

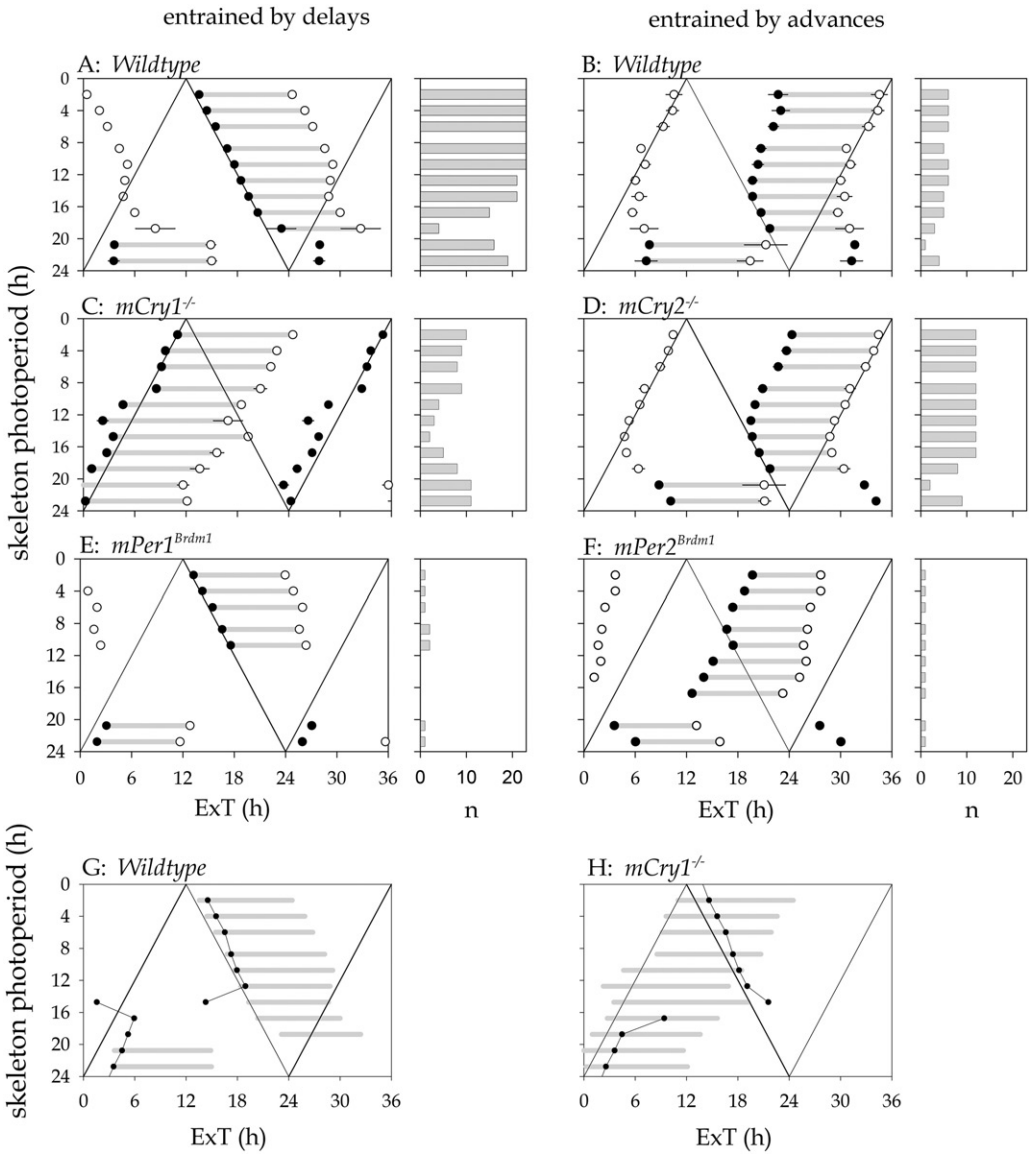

FIGURE 5. (A–F) Entrainment and phase (external time, ExT, see Daan & Merrow, 2002) of mice in changing skeleton photoperiods. The graphs show the onsets (closed dots) and offsets (open dots) of activity (± s.e.m.) with the number of stably entrained individuals (*n*) alongside. Mice entrained by delays (left) with onset locked onto one of the light pulses: (A) 23 wild-type mice; (C) 12 $mCry1^{-/-}$ mice; (E) the two only $mPer1^{Brdm1}$ mutants with a stable phase of entrainment. Mice entrained by advances (right) with offset locked onto one of the pulses: (B) six wild-type mice; (D) 12 $mCry2^{-/-}$ mice; (F) the single $mPer2^{Brdm1}$ mutant where a stable phase of entrainment could be reliably assessed. (G) and (H) Comparison of phase of the average activity (grey bars) in the skeleton photoperiod experiments and the prediction (connected dots) on the basis of a phase only model with fixed α and PRC. Wild-type mice (G) with periods shorter than 24 h (mean $\alpha = 23.75$ h) and PRC as measured by Spoelstra et al. (2004). $mCry1^{-/-}$ mutants (H, mean $\alpha = 22.26$ h) with PRC as measured by (Spoelstra et al., 2004).

To circumvent the confounding effect of masking in the full photoperiod experiment, we carried out a more complete experiment using skeleton photoperiods instead of full photoperiods. Compared with experiment 1, the Zeitgeber strength of the two light pulses in the skeleton photoperiod in experiment 2 was much lower. Under this protocol, most of the *mPer1* and *mPer2* mutants were insufficiently rhythmic and did not express a clear alternation of activity and rest time. Only one *mPer2* mutant mouse was rhythmic and did not show any compression of α. It appeared indeed fully insensitive to the delaying effects of the light pulse occurring around activity onset, and was solely entrained by the advancing pulse around the end of activity. This result is in agreement with our full photoperiod experiment and also with the finding that $mPer2^{Bmrd}$ mice have no (Albrecht et al., 2001) or a reduced (Pendergast et al., 2010; Spoelstra et al., 2004) delay response to light compared with wild-type mice. Moreover, several studies show that $mPer2^{Brdm1}$ τ values are significantly shorter compared with those found in wild-type mice under both constant dark (Spoelstra

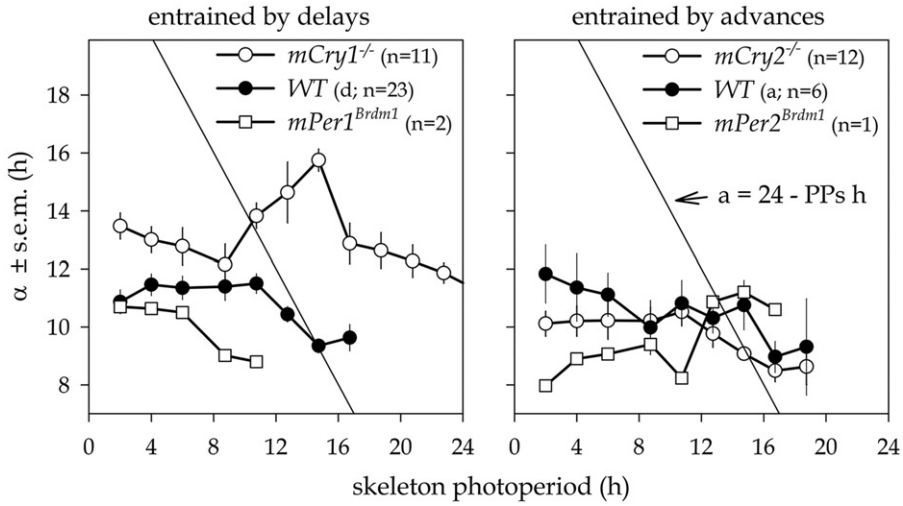

FIGURE 6. α compression. The duration of α averaged per group of mice (±s.e.m.) plotted as a function of increasing skeleton photoperiod, and compared with the length of the initially longest dark interval. α compression occurs between PPs = 10 and 16 in wild type and *mCry2*$^{-/-}$ mice, but is not observed in the other genotypes.

et al., 2004) and constant light (Spoelstra & Daan, 2008; Steinlechner et al., 2002) conditions. Correspondingly, Schwartz et al. (2011) entrained Syrian hamsters to T-cycles of either 23.33 h or 24.67 h duration with one single 1 h light pulse per cycle. The light pulse given in the short T-cycle mimics dawn whereas in the long T-cycle mimics dusk. They found that the ''dawn light pulse'' advanced *Per1* mRNA expression and the ''dusk light pulse'' delayed *Per2* mRNA expression in the SCN concluding that *Per1* is linked to dawn and advances while *Per2* is linked to dusk and delays. In humans, individuals suffering from familial advanced sleep-phase disorder (FASPD) are characterized by the difficulty to phase delay their sleep time onset due to alterations in posttranslational regulation of PER2 protein (reviewed in Chong et al., 2012). In addition, transgenic mice carrying the Per2 mutation that provokes FASPD in humans are characterized by and advanced phase of activity onset and by a shorter activity period length in constant dark conditions compared to wild-type mice (Xu et al., 2007). Hence, data suggesting a key role of PER2 on the ability of organisms to phase delay and period lengthen have been accumulated in the last decade. Muñoz and colleagues showed that under long-term constant light exposure PER2 protein, but not *mPer2* mRNA, is constitutively and highly expressed in the mouse SCN (Muñoz et al., 2005). They hypothesized that probably posttranslational mechanisms such as degradation of PER2 might be inhibited by constant light. Muñoz and co-workers suggested that this is a potential mechanism by which PER2 contributes to phase delays and period lengthening.

To understand the behavior of the other genotypes in this protocol, we first elucidate some general principles in entrainment by skeleton photoperiods. The first is that we usually see better entrainment under extreme PPs than under PPs in the neighborhood of 12 h (Figures 4 and 5). This is readily understood on the basis of the single light pulse PRC. When the two pulses follow each other after a brief dark interval, they hit either the delay part of the PRC – in the case of systems with a short τ – or the advance part of the PRC – in the case of systems with a long period. Thereby, the two pulses tend to reinforce each other and hence constitute a stronger Zeitgeber than two pulses far apart. When two pulses are about 12 h apart, they will have the tendency to hit opposite phases in the PRC and simultaneously generate delays and advances. These will partly offset each other, and effectively constitute a weaker Zeitgeber. Hence, we often see free running rhythms, with evident relative coordination in PPs around 12 h. This is for instance clearly visible in Figure 4(B), where the short τ of a *mCry1*$^{-/-}$ knockout requires a greater daily delay than the symmetrical PPs 12 can offer.

The breakdown of entrainment often occurs at larger PPs than 12. This is when the systems jump over, such that the subjective night settles in the lengthening dark interval when it can no longer be contained in the shorter interval. Hence, the absence of entrainment here is only temporary. The phenomenon highlights another feature specific for a nearly symmetric dark interval. This is the phenomenon of bistability, which has been made understood and fully evaluated by (Pittendrigh, 1966, 1981). Briefly, when the two pulses are exactly 12 h apart in a skeleton photoperiod, the Zeitgeber may be sufficiently strong to lead to stable entrainment by frequency demultiplication. If so, the subjective night can be in either interval because these are identical; it is both determined and fully predicted by initial conditions (Pittendrigh, 1981). A tiny reduction of the interval enclosing the subjective night will not interfere with entrainment, but as the interval gets shorter and shorter a moment will come where the situation is no longer stable. The system will then phase jump its subjective night to the other, longer interval. Assuming that the PRC and endogenous period τ remain the same throughout entrainment one can quantitatively predict at which PPs this will happen. As shown before by Pittendrigh and Daan (1976b) for *Peromyscus leucopus*

and *Mesocricetus auratus* the system hangs much longer with activity in the short interval than predicted (see Figure 5G). Hence, when the two light pulses are closer together, the assumption of a constant PRC is probably violated. The action of the two pulses probably distorts the PRC such that the system no longer acts as a phase-only oscillator. A possible reason for this distortion is that the PPs have affected the internal phase relationship between oscillatory components such as a morning and an evening component. The distortion of the oscillation is also observed in the duration of daily activity α. Compression of α by shortening PPs was clearly visible in wild-type mice, both in those with τ shorter and those with τ longer than 24 h (Figure 6). α Compression also was observed in the *mCry2*$^{-/-}$, but not in *mCry1*$^{-/-}$.

The conclusion from this study is that α compression by long (skeleton) photoperiods is observed in *mCry2*$^{-/-}$ and uncertainly in *mPer1*^{Brdm1}. This refutes prediction from the molecular E/M model (Daan et al., 2001) that defects in these genes render either E or M dysfunctional and thereby abolish α compression. The mutation in the *mPer2* gene, however, clearly prevents α compression. So far, all predictions from the model (PRC level: Pendergast et al., 2010; Spoelstra et al., 2004); τ change with LL (Spoelstra & Daan, 2008) have been upheld for the *mPer2* gene. This reinforces the idea that *mPer2* is specifically involved with a component in the circadian system implicated in the coupling to lights-off, even if the model as a whole does not apply.

ACKNOWLEDGMENTS

We thank U. Albrecht and G. T. J. van der Horst for the supply of founder animals for our transgenic mouse colony, Monique Huizinga for animal care, and maintenance and G. J. F. Overkamp for expert technical help.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper. All experiments are done in accordance with IACUC-RUG protocol 4433A and 2347-4 and with the international ethics standards in chronobiology research compiled by Portaluppi et al. (2010). It was supported by the EC's Fifth Framework Project BRAINTIME (QLRT-2001-01829) and the Sixth Framework Project EUCLOCK (No. 018741).

REFERENCES

Albrecht U, Zheng B, Larkin D, et al. (2001). mPer1 and mPer2 are essential for normal resetting of the circadian clock. J Biol Rhythm. 16:100–4.

Brandstätter R, Kumar V, Abraham U, Gwinner E. (2000). Photoperiodic information acquired and stored *in vivo* is retained *in vitro* by a circadian oscillator, the avian pineal gland. Proc Natl Acad Sci USA. 97:12324–8.

Chong SYC, Ptáček LJ, Fu Y-H. (2012). Genetic insights on sleep schedules: This time, it's personal. Trends Genet. 28:598–605.

Daan S, Albrecht U, van der Horst GTJ, et al. (2001). Assembling a clock for all seasons: Are there m and e oscillators in the genes? J Biol Rhythm. 16:105–16.

Daan S, Merrow M, Roenneberg T. (2002). External time–internal time. J Biol Rhythm. 17:107–9.

Grima BE, Chélot E, Xia R, Rouyer F. (2004). Morning and evening peaks of activity rely on different clock neurons of the Drosophila brain. Nature. 431:869–73.

Hazlerigg DG, Ebling FJP, Johnston JD. (2005). Photoperiod differentially regulates gene expression rhythms in the rostral and caudal SCN. Curr Biol. 15:R449–50.

Illnerová H. (1986). Circadian rhythms in the mammalian pineal gland. Prague: Academia, pp. 1–91.

Inagaki N, Honma S, Ono D, et al. (2007). Separate oscillating cell groups in mouse suprachiasmatic nucleus couple photoperiodically to the onset and end of daily activity. Proc Natl Acad Sci USA. 104:7664–9.

Jagota A, De la Iglesia HO, Scwartz WJ. (2000). Morning and evening circadian oscillations in the suprachiasmatic nucleus *in vitro*. Nat Neurosci. 3:372–6.

Kenagy GJ. (1980). Center-of-gravity of circadian activity and its relation to free-running period in two rodent species. J Interdiscipl Cycle. 11:1–8.

Muñoz M, Peirson SN, Hankins MW, Foster RG. (2005). Long-term constant light induces constitutive elevated expression of mPER2 protein in the murine SCN: A molecular basis for Aschoff's rule? J Biol Rhythm. 20:3–14.

Naito ET, Watanabe T, Tei H, et al. (2008). Reorganization of the suprachiasmatic nucleus coding for day length. J Biol Rhythm. 23:140–9.

Pendergast JS, Friday RC, Yamazaki S. (2010). Photic entrainment of period mutant mice is predicted from their phase response curves. J Neurosci. 30:12179–84.

Picot MP, Cusumano P, Klarsfeld A, et al. (2007). Light activates output from evening neurons and inhibits output from morning neurons in the Drosophila circadian clock. PLoS Biol. 5: 2513–21.

Pittendrigh CS. (1966). The circadian oscillation in Drosophila pseudoobscura pupae: A model for the photoperiodic clock. Z Pflanzenphysiol. 54:S275–307.

Pittendrigh CS. (1981). Circadian systems: Entrainment. In Aschoff J, ed. Handbook of behavioural neurobiology, biological rhythms. New York: Plenum Press, pp. 4:95–124.

Pittendrigh CS, Daan S. (1976a). A functional analysis of circadian pacemakers in nocturnal rodents. I. The stability and lability of spontaneous frequency. J Comp Physiol. 106: 223–52.

Pittendrigh CS, Daan S. (1976b). A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: Pacemaker as clock. J Comp Physiol A. 106:291–331.

Pittendrigh CS, Daan S. (1976c). A functional analysis of circadian pacemakers in nocturnal rodents. V. Pacemaker structure: A clock for all seasons. J Comp Physiol. 106: 333–55.

Portaluppi F, Smolensky MH, Touitou Y. (2010). Ethics and methods for biological rhythm research on animals and human beings. Chronobiol Int. 27:1911–29.

Rieger D, Shafer OT, Tomioka K, Helfrich-Förster C. (2006). Functional analysis of circadian pacemaker neurons in *Drosophila melanogaster*. J Neurosci. 26:2531–43.

Spoelstra K, Albrecht U, van der Horst GTJ, et al. (2004). Phase responses to light pulses in mice lacking functional per or cry genes. J Biol Rhythm. 19:518–29.

Spoelstra K, Daan S. (2008). Effects of constant light on circadian rhythmicity in mice lacking functional cry genes: Dissimilar from per mutants. J Comp Physiol A. 194:235–42.

Steinlechner S, Jacobmeier B, Scherbarth F, et al. (2002). Robust circadian rhythmicity of Per1 and Per2 mutant mice in constant light, and dynamics of Per1 and Per2 gene expression under long and short photoperiods. J Biol Rhythm. 17:202–9.

Stoleru D, Nawathean P, De la paz Fernández M, et al. (2007). The Drosophila circadian network is a seasonal timer. Cell. 129: 207–19.

Stoleru D, Peng Y, Agosto J, Rosbash M. (2004). Coupled oscillators control morning and evening locomotor behaviour of Drosophila. Nature. 431:862–8.

Stoleru D, Peng Y, Nawathean P, Rosbash M. (2005). A resetting signal between Drosophila pacemakers synchronizes morning and evening activity. Nature. 438:238–42.

Schwartz WJ, Tavakoli-Nezhad M, Lambert CM, et al. (2011). Distinct patterns of period gene expression in the suprachiasmatic nucleus underlie circadian clock photoentrainment by advances or delays. Proc Natl Acad Sci USA. 108:17219–24.

Sumová A, Illnerová H. (1998). Photic resetting of intrinsic rhythmicity of the rat suprachiasmatic nucleus under various photoperiods. Am J Physiol-Reg I. 274:R857–63.

Van der Horst GTJ, Muijtjens M, Kobayashi K, et al. (1999). Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature. 398:627–30.

Wehr TA, Aeschbach D, Duncan WC. (2001). Evidence for a biological dawn and dusk in the human circadian timing system. J Physiol. 535:937–51.

Xu Y, Toh KL, Jones CR, et al. (2007). Modeling of a human circadian mutation yields insights into clock regulation by PER2. Cell. 128:59–70.

Yoshii T, Funada Y, Ibuki-Ishibashi T, et al. (2004). Drosophila cry b mutation reveals two circadian clocks that drive locomotor rhythm and have different responsiveness to light. J Insect Physiol. 50:479–88.

Zheng B, Larkin DW, Albrecht U, et al. (1999). The mPer2 gene encodes a functional component of the mammalian circadian clock. Nature. 400:169–73.

Zheng B, Albrecht U, Kaasik K, et al. (2001). Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell. 105:683–94.